Mass-based fraction collection of synthetic peptides using the Agilent 1100 Series purification system

Application

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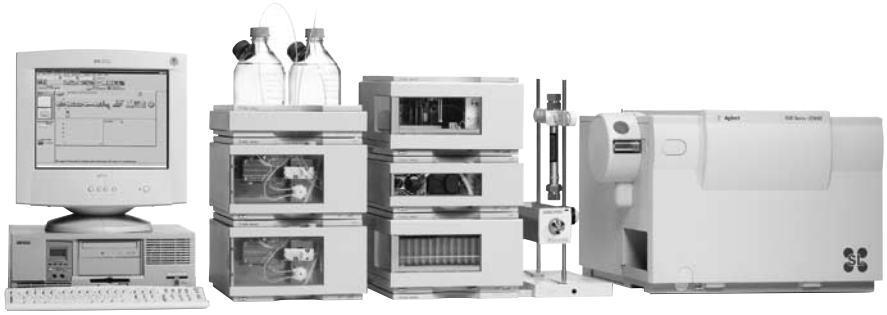

Abstract

Synthetic peptides are becoming more and more important as drug candidates in the treatment of a variety of diseases. In order to keep pace with the growing number of newly synthesized peptides, peptide purification should not represent the bottleneck in the drug discovery process. Mass-based fraction collection is an efficient technique for the purification of compounds with well-known masses. In contrast to fraction triggering with less specific detectors, only the compound of interest is purified in each run. Hence, it is not necessary to pick target compounds out of a series of redundant fractions that have been collected. This Application Note demonstrates mass-based purification of a series of synthetic peptides with the Agilent 1100 Series purification system. The modular set-up of the system, a reliable fully-automated delay volume calibration and a comprehensive software package provide a versatile platform for purification tasks up to 100 mL/min. Crude peptides were successfully purified by reverse phase HPLC from less than 1 kDa to more than 10 kDa, covering a pI range from 4 to 13.

Agilent Technologies

Introduction

Today, solid phase peptide synthesis is the method of choice for large-scale peptide production. In order to remove scavengers and reaction by-products a subsequent purification step is indispensable. Usually preparative reverse phase HPLC is applied to purify crude peptides after synthesis. In such an approach target peptides are UV-based fractionated and their identity and purity is determined offline by mass spectrometry (MS) e.g. MALDI-TOF. However, this approach bares some weak points. Since UV detection is not selective in each chromatographic run several peaks might be collected and the target compound has to be picked out of a series of redundant fractions (figure 1A). Moreover, fractions are collected even though a synthesis might not have been successful. After chromatography some preparation steps are required for MS investigation of the fractions. In summary, UV-based fraction collection with subsequent offline MS-analysis is time-consuming, wastes valuable resources in the fraction collector and does not provide any online MS information.

Another very efficient approach for the purification of compounds with well-known masses is mass-based fraction collection. The benefits of this technique are illustrated in figure 1.

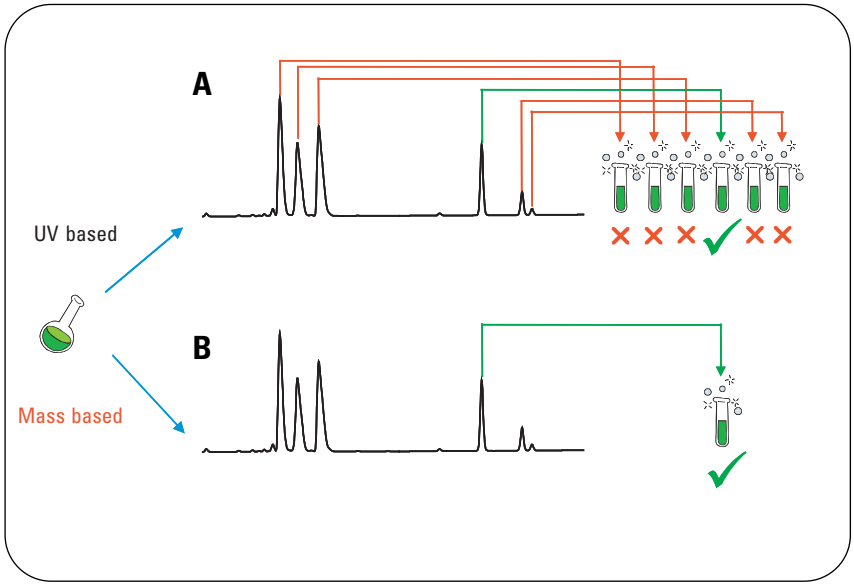

Figure 1
UV-based (A) versus mass-based (B) fraction collection

Equipment

The instrumental set-up of the Agilent 1100 Series purification systems is as follows:

- two Agilent 1100 Series preparative pumps
- Agilent 1100 Series isocratic pump
- Agilent 1100 Series preparative autosampler
- Agilent 1100 Series column organizer
- Agilent 1100 Series diode array detector (DAD) with 0.03 mm flow cell
- Agilent active splitter
- Agilent 1100 Series high performance LC/MSD SL with API-Electrospray source
- Agilent 1100 Series fraction collector PS

The system was controlled using the Agilent ChemStation (rev. A.09.03) and the purification/high-throughput software (rev. A.01.02) add-on software. For further information about the Agilent 1100 Series purification system refer to reference 1. Figure 2 shows the instrumental set-up comprising two flow paths.

Dispersion

An often-overlooked phenomenon that impacts the compound distribution during migration from the detector to the fraction collector is dispersion. Dispersion equates with peak broadening and the main flow leads from the pump(s) to the autosampler, the column, the DAD and the active splitter before reaching the fraction collector. Since the MSD is a destructive detector and the flow rate of the main flow is too high to route it directly into the electrospray source, a make-up flow is sustained by an isocratic pump. This make-up flow leads from the isocratic pump to the active splitter before reaching the MSD. The Agilent active splitter is a key component of the purification system. This splitter functions as is illustrated in figure 3. Small aliquots of the HPLC main flow stream are transferred to a mass spectrometer flow stream. In contrast to a traditional splitter design the Agilent active splitter offers several advantages. The splitter is fully software-controlled. Split ratios ranging from about 10:1 to 300,000:1 can easily be adjusted without any backpressure. The set split ratios are highly accurate and constant and are not being affected by changes in viscosity, temperature and tubing length as with traditional splitters.

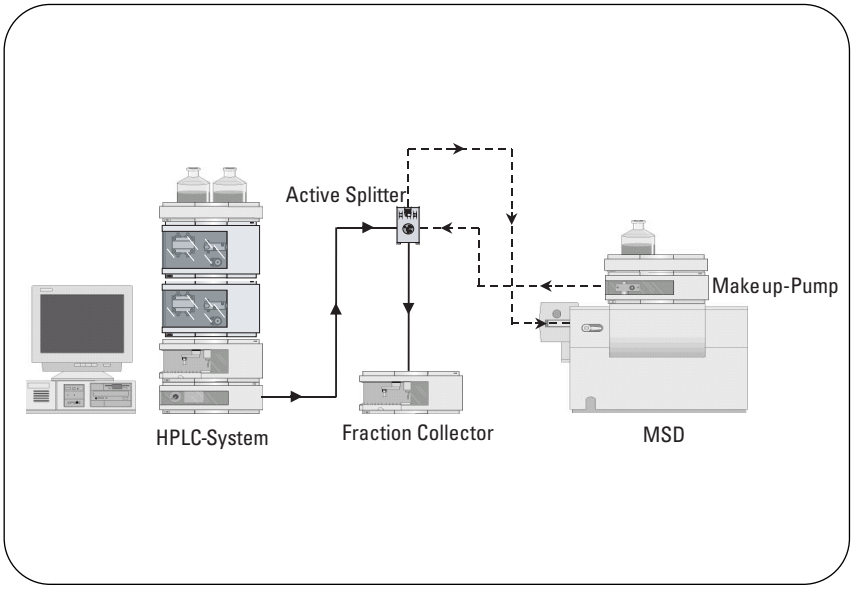

Figure 2
Instrumental set-up of mass-based purification system

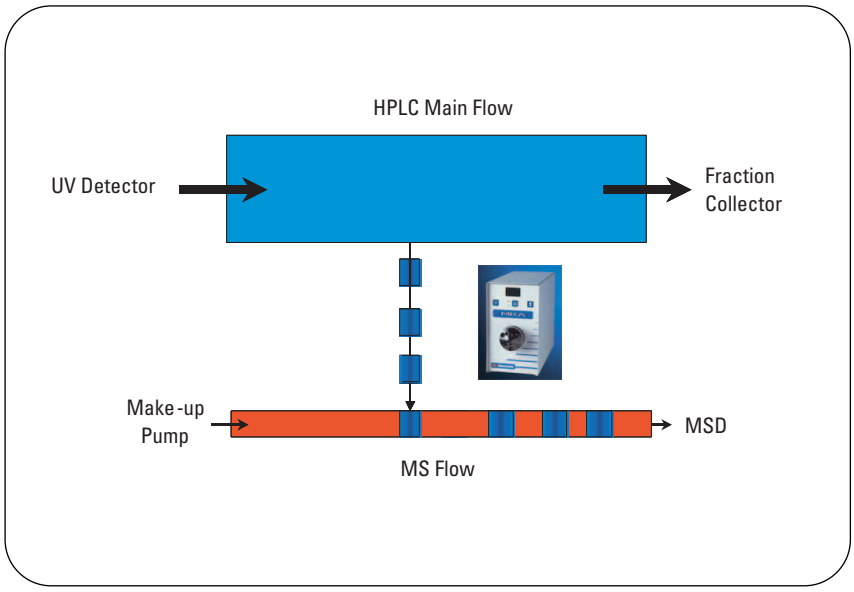

Figure 3
Functional principle of the Agilent active splitter

Moreover, the active splitter does not add any significant delay volume to the system and therefore keeps dispersion low (for further information please refer to reference 2). In peptide purification usually TFA as an ion-pairing agent gives the best results regarding compound separation. However, TFA is known to suppress ion formation and therefore gives rise to poor MSD sensitivity. On the other hand, using formic acid as an ion-pairing agent leads to good signals in the MSD but the compound separation efficiency on reverse phase columns is usually not as good as with TFA. In order to take advantage of both ion-pairing reagents TFA (0.08 %) was added to the HPLC main flow whereas formic acid (0.1 %) was added to the make-up flow. Since only a small aliquot of the HPLC main flow is transferred to the MS flow, transferred TFA molecules become diluted. This effect is illustrated in figure 4. In both total ion current (TIC) chromatograms the same RNase A sample was subject to sensitivity tests. In the upper chromatogram 0.1 % TFA was added to the make-up flow, in the lower chromatogram 0.1 % formic acid was added to the make-up flow. In both cases 0.1 % TFA was added to the main flow for best chromatographic separation, the split-ratio remained the same. The improvement in sensitivity when using formic acid instead of TFA in the make-up flow is clearly visible in the lower panel.

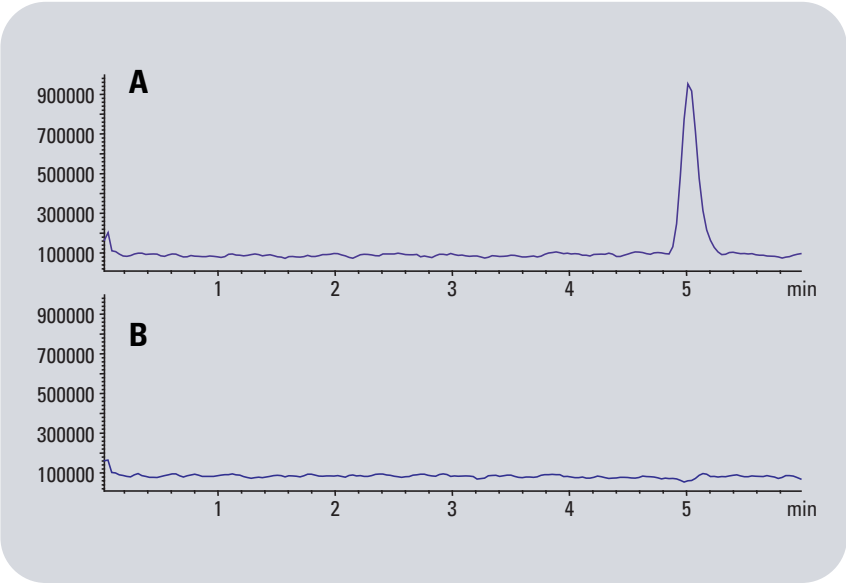

Figure 4
TIC signal when using 0.1 % TFA (A) and 0.1 % formic acid (B) in the make-up flow

Experimental

Sample preparation

After synthesis at Cancer Research, UK the crude peptide samples were lypholized and sent to Agilent Technologies, Waldbronn. The lypholized samples were then re-dissolved and filtered (0.2 µm) prior to chromatography. Appropriate solvent conditions (water, acetonitrile, TFA) for each sample were chosen according to pI and hydrophobicity of the corresponding peptides. The final peptide concentrations were between 5 and 10 mg/mL.

Mass-based fraction collection

Mass-based fraction collection was either triggered on the singly charged state of the target peptide or on one of the multiply charged states. In order to yield a pure product for peak triggering the MS signal was connected to an UV-signal via a logical "*and*". In particular, this means that a peak is only collected when the predefined trigger conditions for threshold and slope for both detectors are fulfilled. In order to avoid collection of invalid peaks due to fluctuations in the baselines of both trigger signals, threshold values for all signals were specified.

Results and Discussion

Purification

Figure 5 shows the chromatogram of a preparative scale purification of an acidic (pI 4.3) 56 amino acid (6021 Da) peptide at 25 mL/min. Fraction collection was triggered at the UV signal at 214 nm with a threshold of 15 mAU and an m/z of 1205.3 (the TIC is shown in the insert). The vertical lines indicate beginning and end of fraction collection, respectively. Additionally, the position of the collected fraction in the fraction collector and the target mass are given. The horizontal line visualizes the UV threshold value for fraction collection. All conditions for fraction collection were fulfilled as soon as the UV-signal at 214 nm exceeded the threshold value of 15 mAU and the target m/z of 1205.3 was detected by the MSD. Figure 6 shows the signals that are generated by the MSD. The blue curve is the total ion current (TIC) chromatogram which is a superposition of all ions being detected by the MSD in the specified range. In contrast, the extracted ion chromatogram (EIC, black curve) just shows the signal that is solely produced by the target m/z of 1205.3

Re-analysis

After purification the fractions were re-analyzed applying the analytical method mentioned above. Figure 7 shows the TIC chromatogram of the reanalyzed purified peptide. The insert shows the

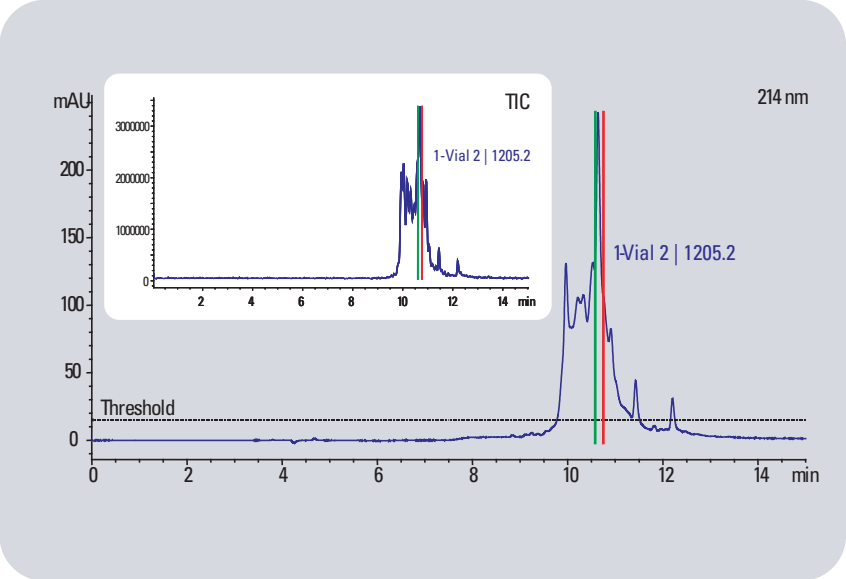

Figure 5
UV-signal at 214 nm and TIC chromatogram (insert) of the purification process. The vertical lines indicate start and end of fraction collection.

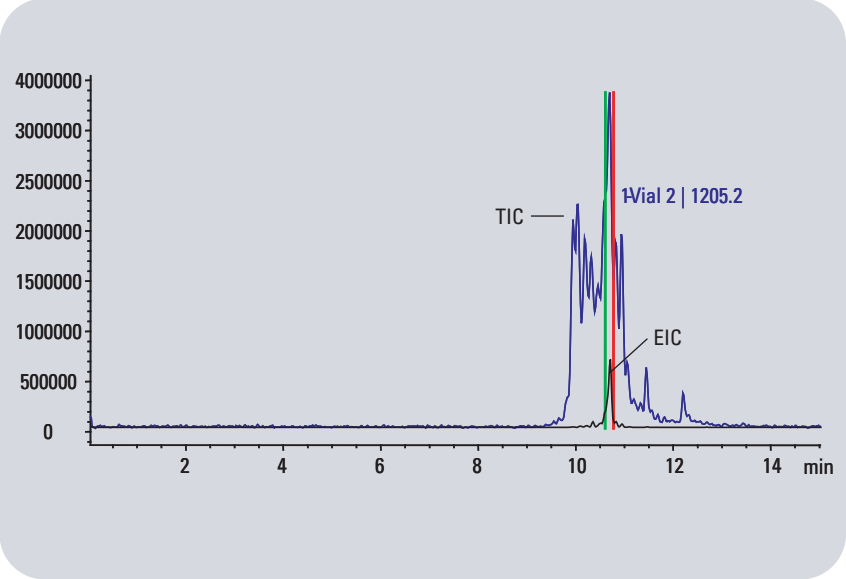

Figure 6
TIC and the underlayed target ion EIC signal of the purification run.

ESI mass spectrum that was extracted from the TIC chromatogram. Deconvolution of the mass spectrum results in a mass of 6021 Da. Chromatogram as well as spectrum show that purification was successful. For all peptides a recovery between 90 and 100 % could be achieved.

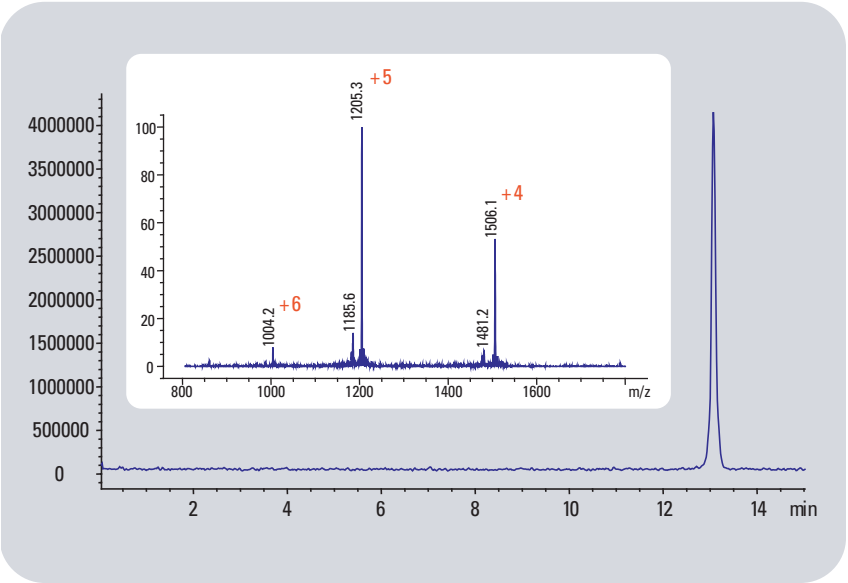

Figure 7
TIC signal and extracted ESI mass spectrum (insert) of the re-analysis. The numbers indicate ion charges.

Conclusion

Mass-based fraction collection is the right choice for compound purifications when the target masses are known. Besides the purification of combinatorial chemistry libraries[3] mass-based fraction collection is also an elegant method for purification of synthetic peptides. In our collaboration with Cancer Research UK we could show that the Agilent 1100 Series purification systems can cope with a wide variety of peptides. We could successfully purify hydrophobic and hydrophilic peptides that differ in pI between 4 and 13 and cover a mass-range between 1 and more than 10 kDa. Furthermore, we pointed out that fraction collection triggered by predefined masses is advantageous over conventional less specific detectors. Since only the compounds of interest are being collected in each run no additional time needs to be spent to pick target compounds out of a series of redundant fractions. Therefore, mass-based fraction collection is highly efficient and saves valuable resources. Furthermore, we could show that when coupling ESI-MS to HPLC for data evaluation no additional preparation steps are needed as for offline MS techniques. MS information can easily be analyzed online during a chromatographic run. Further, our findings indicate that peptide characterization with an ESI-MSD is an alternative to offline MS methods e.g. MALDI-TOF.

References

1.
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